Interaction of Environmental Chemicals with the Estrogen and Progesterone Receptors from the Oviduct of the American Alligator

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Reports of reproductive abnormalities in the American alligator from Lake Apopka, Florida, have been linked to a spill of DDT and other pesticides suspected of having hormonelike activity. To determine whether environmental chemicals had the potential to function as exogenous hormones in the American alligator, we examined the ability of chemicals to bind the estrogen receptor (aER) and progesterone receptor (aPR) in a protein extract prepared from the oviduct of the alligator. In competition binding assays with [^{3}H]17β-estradiol, some DDT metabolites showed inhibition of [^{3}H]17β-estradiol binding to aER. A combination of DDTs demonstrated an additive decrease in [^{3}H]17β-estradiol binding to aER. Modern-use chemicals such as alachlor, *trans*-nonachlor, endosulfan, and atrazine also competed with [^{3}H]17β-estradiol for binding to the aER. To test the effect of chemicals identified in alligator eggs from Lake Apopka on [^{3}H]17β-estradiol binding, we mixed these chemicals at concentrations measured in eggs in the competition binding assay. 2,2-bis(4-chlorophenyl)-N-(methoxymethyl)acetamide (*p,p′*-DDD) and *trans*-nonachlor, both found in Lake Apopka, interacted with aER, whereas others such as chlordane and toxaphene did not. Surprisingly, combinations of these chemicals decreased [^{3}H]17β-estradiol binding in a greater than additive manner. To assess the ability of chemicals to interact with aPR, we performed competition binding assays with the synthetic progestin [^{3}H]R5020. Most of the chemicals tested did not reduce [^{3}H]R5020 binding to aPR, whereas endosulfan, alachlor, and kepone inhibited binding. These results provide the first evidence that environmental chemicals bind the aER and aPR from the American alligator, supporting the hypothesis that the reported reproductive abnormalities may be related to the modulation of endocrine-related responses. The findings that combinations of chemicals demonstrated a greater than additive interaction with the aER and some chemicals bind to the aPR in the competition binding assay are novel. This suggests that interactions of these chemicals with the endocrine system are complex. *Key words*: alligator, DDT, environmental estrogens, estrogen receptor, pesticides, progesterone receptor. *Environ Health Perspect* 104:1318–1322 (1996)

A major pesticide spill in 1980, composed mainly of synthetic chemicals including the pesticides DDT and dicofol, coincided with a decline in the population of juvenile alligators from Florida's Lake Apopka (*1*). Hatch rates of alligator eggs from this lake were markedly reduced and the reproductive tract morphology and hormone levels of surviving juvenile alligators were seriously disrupted. Morphological effects were characterized by poorly organized testes and abnormally small phalli in juvenile males and large numbers of polyovular follicles and multinucleated oocytes in females (*2,3*). Prenatal exposure of the Lake Apopka alligators to environmental chemicals functioning as hormones has been proposed as the mechanism behind the loss of viability of the alligator eggs and the reproductive abnormalities seen in the juvenile alligators (*2*).

It has been suggested that the abnormalities observed in these alligators are similar to those reported following embryonic exposure to the potent synthetic estrogen diethylstilbestrol (DES) (*4*). Prenatal exposure to DES in both humans and experimental animals has been associated with an array of reproductive abnormalities (*5,6*). Effects of embryonic exposure to DES in female mice include vaginal clear-cell carcinoma, reproductive dysfunction, and abnormalities of the uterus and oviducts (*7–10*), whereas male mice exhibited testicular abnormalities and feminization of reproductive organs (*11*). DES exposure has also been shown to have estrogenic effects in nonmammalian species. In chick embryos, DES has been shown to induce expression of the estrogen-responsive gene ovalbumin and promote morphogenesis in the embryonic Müllerian ducts (*12*). Since alligators are also a nonmammalian organism, the results with DES in chick embryos suggest that synthetic estrogens may also function in alligators.

It has been demonstrated that numerous synthetic chemicals, in addition to DES, can have hormonelike activity in whole animal studies and *in vitro* assays (*13*). Alligator eggs collected from Lake Apopka contained measurable concentrations of 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene

(*p,p′*-DDE), 2,2-bis(4-chloropenyl)-N-(methoxymethyl)acetamide (*p,p′*-DDD), *trans*- and *cis*-nonachlor, polchlorinated biphenyls, dieldrin, toxaphene, and chlordane (*14*). *p,p′*-DDD and *cis*- and *trans*-nonachlor have estrogenic activity in MCF-7 human breast cancer cells (*15*). *p,p′* DDE has been shown to be an antiandrogen in the rat (*16*). Toxaphene and dieldrin, but not chlordane, stimulated the proliferation of MCF-7 cells in the E-SCREEN assay (*17*).

We have previously identified receptors for 17β-estradiol and progesterone in the alligator oviduct (*18*). The alligator estrogen receptor (aER) displays a saturable, single component, high affinity binding activity for 17β-estradiol with a dissociation constant (K_d) of 0.5 nM. The alligator progesterone receptor (aPR) has a saturable, single component, high affinity binding activity with a K_d of 0.9 nM for the synthetic progestin R5020. To test the interaction of environmental chemicals with the aER and aPR, including those found in Lake Apopka, we prepared a protein extract from the oviductal tissues of the American alligator. Numerous chemicals competed with [^{3}H]17β-estradiol for binding to the aER. Fewer chemicals competed with the synthetic progestin [^{3}H]R5020 for binding to the aPR. Collectively, these data indicate that some environmental chemicals, including those found in the alligator eggs of Lake Apopka, interact with the aER and/or aPR.

Materials and Methods

17β-[3,4,6,7-^{3}H] estradiol (84 Ci/mmol), [^{3}H]R5020 (84 Ci/mmol), and radioinert R5020 were obtained from DuPont NEN Products (Boston, MA). Radioinert 17β-estradiol and 1,1,1-trichloro-2-(p-chlorophenyl)-2-(o-chlorophenyl)ethane,

(*o,p'*-DDT) were purchased from Sigma Chemical Co. (St. Louis, MO). 2-2-bis(4-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (*o,p'*-DDD); 2,2-bis(*p*-chlorophenyl)-ethanol (DDOH); 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (*o,p'*-DDE); 1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane (*p,p'*-DDT); *p,p'*-DDD; *p,p'*-DDE; 2,2-bis(*p*-chlorophenyl)-acetic acid (*p,p'*-DDA); and methoxychlor were purchased from Aldrich Chemical Company (Milwaukee, WI). Dicofol, *trans*-nonachlor, kepone, *cis*-nonachlor, endosulfan I, endosulfan II, endosulfan sulfate, 2,4-dichlorophenoxyacetic acid (2,4-D), cyanazine, atrazine, dieldrin, and alachlor were purchased from AccuStandard (New Haven, CT). Aroclor 1242, chlordane, and toxaphene were purchased from Supelco (Bellefonte, PA). All chemicals were dissolved in DMSO except for atrazine and cyanazine, which were dissolved in methanol; alachlor, which was dissolved in ethanol; toxaphene and chlordane, which were dissolved in isooctane; and 2,4-D acid, which was dissolved in 20 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM ethyleneglycoltetraacetic acid (EGTA). All chemicals were at least 99% pure, with the exceptions of methoxychlor (95%), kepone (98%), *cis*-nonachlor (98.9%), cyanazine (97%), and toxaphene (purity not available).

To prepare a protein extract for estrogen-binding assays, frozen oviductal tissue from adult female alligators (*Alligator mississippiensis*) captured from several lakes in central Florida was thawed on ice and placed into homogenization buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM NaVO$_4$, 10 mM NaPO$_4$, 50 mM NaF, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 2 mg/ml pepstatin, and 50 µl/ml aprotin). The tissue was minced by a Polytron homogenizer and centrifuged at 4,000 rpm for 10 min at 4°C. The supernatant was collected and brought to 400 mM with KCl and incubated at 4°C for 15 min. The supernatant was centrifuged at 15,000 rpm for 10 minutes at 4°C and the protein extract was flash frozen on dry ice.

All [^{3}H]17β-estradiol binding assays used 35 µl of cytosol dissolved in 165 µl homogenization buffer at 25°C. For the competition binding assays, alligator protein extract was incubated with 2.5 nM [^{3}H]17β-estradiol for 1 hr at 25°C. Free [^{3}H]17β-estradiol was removed by incubation with chardex (5% activated charcoal and 0.5% dextran dissolved in homogenization buffer) for 10 min at 4°C and centrifugation for 3 min at 15,000g. Samples were then incubated with either vehicle or increasing concentrations of radioinert environmental chemicals at 25°C for 1 hr. The concentration of

ethanol or DMSO did not exceed 1% in the reactions. Free [^{3}H]17β-estradiol was again removed by incubation with chardex for 10 min at 4°C and centrifugation for 3 min at 15,000g. The bound [^{3}H]17β-estradiol was measured by scintillation counting. The data are representative of at least three independent experiments with three replicates.

[^{3}H]R5020 binding assays used 10 µl cytosol dissolved in 190 µl homogenization buffer (plus 1 mM dithiothreitol and 10 mM sodium molybdate) at 4°C. For the competition binding assays, the protein extract was incubated with 2.5 nM [^{3}H]R5020 in the presence or absence of increasing concentrations of radioinert chemicals at 4°C for 12 hr. Free [^{3}H]R5020 was removed by incubation with chardex for 10 min at 4°C and centrifugation for 3 min at 15,000g. The bound [^{3}H]R5020 was measured by scintillation counting. The data are representative of at least three independent experiments with three replicates.

Statistics were computed by one-way analysis of variance (ANOVA) least significant difference test (SPSS, Chicago, Illinois). Significant differences in the inhibition of [^{3}H]17β-estradiol or [^{3}H]R5020 binding by environmental chemicals were defined when $p < 0.05$.

Results

Inhibition of [^{3}H]17β-estradiol binding to aER by environmental chemicals. A protein extract prepared from the oviduct of the American alligator was incubated with [^{3}H]17β-estradiol and increasing concentrations of radioinert 17β-estradiol, *o,p'*-DDT, *p,p'*-DDT, *o,p'*-DDD, *p,p'*-DDD, *o,p'*-DDE, *p,p'*-DDE, *p,p'*-DDA, or DDOH. The most effective inhibitor of [^{3}H]17β-estradiol binding to aER was 17β-estradiol with an IC$_{50}$ (the concentration of chemical necessary to inhibit [^{3}H]17β-estradiol binding by 50%) of 7.8 nM (Table 1, Fig. 1A). *o,p'*-DDT, one of the first chemicals identified with estrogenic activity, had an IC$_{50}$ of 9.1 µM. The IC$_{50}$ for *o,p'*-DDT was approximately 1,000-fold higher than the IC$_{50}$ for estradiol, which is consistent with previous competition binding assays using rodent and human ER (*19,20*). *o,p'*-DDD was the most effective DDT metabolite at reducing [^{3}H]17β-estradiol binding, with an IC$_{50}$ of 2.26 µM or approximately 300-fold higher IC$_{50}$ than estradiol. DDOH, *o,p'*-DDE, and dicofol were also able to compete for [^{3}H]17β-estradiol binding, with IC$_{50}$ values ranging between 11.1 and 45.6 µM. The DDT metabolites *p,p'*-DDT, *p,p'*-DDD, and *p,p'*-DDE reached their solubility limit before their IC$_{50}$ values could be determined. *p,p'*-DDA and methoxychlor, which has been shown to require conversion

Chemical	aER binding IC$_{50}$ (µM)
17β-Estradiol	0.0078
o,p'-DDD	2.26
o,p'-DDT	9.1
DDOH	11.1
o,p'-DDE	37.25
Dicofol	45.6
p,p'-DDT	>50[a]
p,p'-DDD	>50[a]
p,p'-DDE	>50[a]
p,p'-DDA	NS
Methoxychlor	NS
trans-Nonachlor	10.6
Cyanazine	19
Atrazine	20.7
Alachlor	27.5
Kepone	34
Aroclor 1242	37.2
cis-Nonachlor	40
Endosulfan I	>50[a]
Endosulfan sulfate	>50[a]
Dieldrin	NS
Endosulfan II	NS
Toxaphene	NS
2,4-D	NS

Abbreviations: IC$_{50}$, concentration that inhibits 50%; DDOH, 2,2-bis(*p*-chlorophenyl)-ethanol; *p,p'*-DDA, 2-2-bis (*p*-chlorophenyl)-acetic acid; 2,4-D, 2,4-(dichlorophenoxy)acetic acid; NS, not significant. Competitive binding assays were performed as described in Materials and Methods. The IC$_{50}$ values were calculated by plotting the percent [^{3}H]17β-estradiol bound versus concentration of environmental chemicals as shown in Figure 1A and B. The data shown are representative of at least three independent experiments with three replicates. Chemicals were tested to their solubility limits. Endosulfan I, dieldrin, and toxaphene had similar binding characteristics for both the aER and the human estrogen receptor (hER) (*31*). [a]Compounds that inhibited [^{3}H]17β-estradiol but were insoluble at concentrations necessary to achieve 50% inhibition.

in vivo to its active metabolite 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) (*21*), did not interact with aER.

The binding of other environmental chemicals was also investigated in the competition binding assay (Table 1, Fig. 1B). Alachlor had an IC$_{50}$ of 27.5 µM, whereas *trans*-nonachlor and *cis*-nonachlor had IC$_{50}$ values of 10.6 µM and 40 µM, respectively. Endosulfan, dieldrin, toxaphene, and chlordane had no appreciable interaction with aER.

The effect of combinations of environmental chemicals in competition binding assays. To test whether mixtures of environmental chemicals were additive, synergistic, or antagonistic, metabolites of DDT at 1 µM were incubated individually or in combination with oviductal extract and [^{3}H]17β-estradiol. Dicofol, *p,p'*-DDD, or *p,p'*-DDE
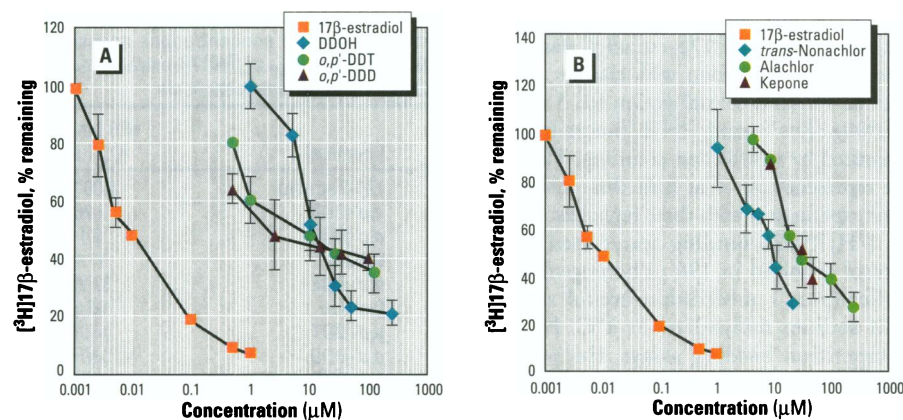
Figure 1. Competition binding assay using environmental chemicals with aER. An oviductal extract containing aER was incubated with [³H]17β-estradiol and varying concentrations of estradiol, 2,2-bis (p-chlorophenyl)-ethanol (DDOH), o,p'-DDT, o,p'-DDD, or p,p'-DDT (A) or estradiol, trans-nonachlor, alachlor, kepone, or dicofol (B), as described in the Materials and Methods. The limit for the solubility of these chemicals was between 100–250 μM in this assay. The data are the mean of three independent experiments with three replicates. Error bars indicate standard error.

Figure 2. The effect of mixtures of DDT and metabolites in the competition binding assay. An oviductal extract containing aER was incubated with [³H]17β-estradiol with 1 μM of dicofol, p,p'-DDD, p,p'-DDE, or o,p'-DDT individually or in combination as described in the Materials and Methods. The limit for the solubility was between 100–250 μM in this assay. The data are the mean of three independent experiment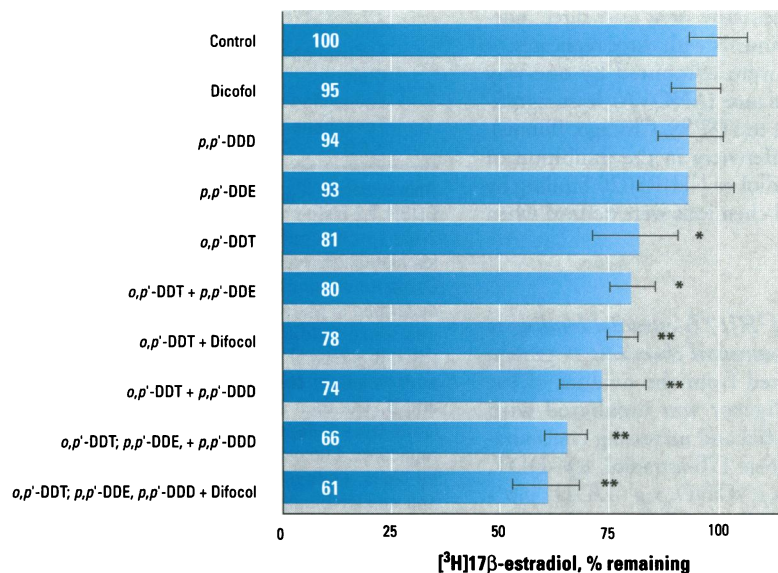s with three replicates. Error bars indicate standard error. *p<0.05 indicates significant inhibition of binding compared to control value. **p<0.05 indicates significant inhibition of binding compared to control and individual treatments with DDT metabolites.

individually did not significantly reduce [³H]17β-estradiol binding, whereas o,p'-DDT inhibited binding by 18% (Fig. 2). A mixture of all four chemicals resulted in a 40% loss of [³H]17β-estradiol, indicating that the decrease in binding was additive.

The effect of mixtures of chemicals was also analyzed by using the seven chemicals identified in alligator eggs from Lake Apopka, Florida (14). These chemicals were incubated individually or in combination, at concentrations measured in alligator eggs with oviductal extract and [³H]17β-estradiol. The seven chemicals alone did not reduce the [³H]17β-estradiol binding to a measurable extent, with

the exception of 2.6 μM p,p'-DDD, which decreased binding by 20% (Fig. 3A). A combination of all seven chemicals reduced [³H]17β-estradiol binding by approximately 60% (Fig. 3B). Toxaphene, dieldrin, and chlordane alone had no effect on [³H]17β-estradiol binding. Surprisingly, the addition of toxaphene, dieldrin, and chlordane to p,p'-DDE, p,p'-DDD, trans-nonachlor, cis-nonachlor, and alachlor resulted in a further 14% decrease in [³H]17β-estradiol binding.

Inhibition of [³H]R5020 binding to aPR by environmental chemicals. To assess the ability of environmental chemicals to bind the aPR, the oviductal extract was incu-

bated with [³H]R5020 and 30 μM radioinert R5020, o,p'-DDT, p,p'-DDT, o,p'-DDD p,p'-DDD, o,p'-DDE, p,p'-DDE, p,p'-DDA, or DDOH. Radioinert R5020 was the most effective chemical at competing with [³H]R5020 for binding to aPR, reducing it to 36% of control (Fig. 4A). DDOH reduced [³H]R5020 binding by almost 25%, but the other DDT metabolites did not significantly reduce [³H]R5020 binding.

Several modern-use chemicals were also tested for interaction with aPR in the competition binding assay. Atrazine, cyanazine, endosulfan sulfate, and dicofol reduced [³H]R5020 binding by 40–50% (Fig. 4B). Interestingly, endosulfan I and II did not inhibit [³H]R5020 binding. Kepone reduced [³H]R5020 binding by almost 60%.

Discussion

We have demonstrated that the aER and aPR prepared from the oviduct of the American alligator were capable of recognizing environmental chemicals, some of which have been reported to have estrogenic activity in whole animal studies or *in vitro* assays (15). Mixtures of DDT metabolites reduced estradiol binding to aER in an additive fashion, whereas combinations of chemicals identified in Lake Apopka inhibited binding in a greater than additive manner. Some chemicals that bound the aER also interacted with the aPR.

One of the earliest examples of environmental chemicals having estrogenic activity was DDT, which was banned from use in the United States in 1972 (22–24). Many top-level predators such as alligators have been reported to contain high concentrations of DDT metabolites in their bodies (25). DDT and several of its metabolites have been shown to be estrogenic in rodents and human mammary carcinoma cells (17,22,23,26). The ability of DDT to interact with the aER suggests that it may also have estrogenic effects in alligators. The ability of the aER to recognize DDT and its metabolites indicates the need for future research to determine if these are active in estrogen-sensitive tissues of the alligator.

Modern-use chemicals, including alachlor, trans-nonachlor, and cis-nonachlor, are some of the most effective chemicals at displacing [³H]17β-estradiol from the aER, as shown in Table 1. Atrazine and cyanazine also displaced [³H]17β-estradiol from the aER. Atrazine and cyanazine have been shown not to possess estrogenic or antiestrogenic activity in the rodent or with the human ER (hER) using *in vitro* assays (17,27–29). These data suggest the potential for differential binding of environmental chemicals with the ER from various species.

Since alligator eggs from Lake Apopka contained numerous environmental chemicals (*14*), we wanted to assess the ability of these chemicals, in the combinations and concentrations found in the eggs, to interact with the aER. Our results suggest that combinations of the chemicals identified in the alligator eggs can inhibit binding of [^{3}H]17β-estradiol by 60% at environmental concentrations (see Fig. 3B). Moreover, these combinations of environmental chemicals appear to have greater than additive effects in the competition binding assay. The environmental chemicals identified in alligator eggs, with the exception of *p,p'*-DDD, demonstrated no displacement of [^{3}H]17β-estradiol individually when tested at the concentrations found in the alligator eggs. However, these chemicals showed significant displacement of [^{3}H]17β-estradiol when assayed in combination.

In contrast, combinations of DDT metabolites inhibited [^{3}H]17β-estradiol binding in an additive fashion. These data may suggest that the mechanism for competition for [^{3}H]17β-estradiol binding is different between the DDT metabolites and other environmental chemicals. Nonetheless, the effect of combinations of environmental chemicals will need to be tested using a functional assay to determine if the greater than additive binding of some environmental chemicals to the aER correlates with increased estrogenic activity.

There have been reports of greater than additive effects of estrogenic compounds interacting with the hER. The growth of

estrogen-responsive MCF-7 cells is stimulated in the presence of some chemical combinations in a greater than additive manner (*30*). Recently, our laboratory has demonstrated that the combinations of the environmental chemicals endosulfan, dieldrin, toxaphene, and chlordane induced hER-mediated transcription of an estrogen-sensitive reporter in yeast in a synergistic manner (*31*). In addition, we have shown that this synergistic activation of the hER correlated with a greater than additive displacement of [^{3}H]17β-estradiol using competition-bind-

ing assays. These results and the observations that the same combinations of chemicals at similar concentrations produced a synergistic inhibition of [^{3}H]17β-estradiol binding to aER suggests that the binding mechanism of the ER from humans and alligators may be conserved. These studies suggest the estrogenic potential of some chemical combinations may be important for determining their ability to modulate endocrine responses.

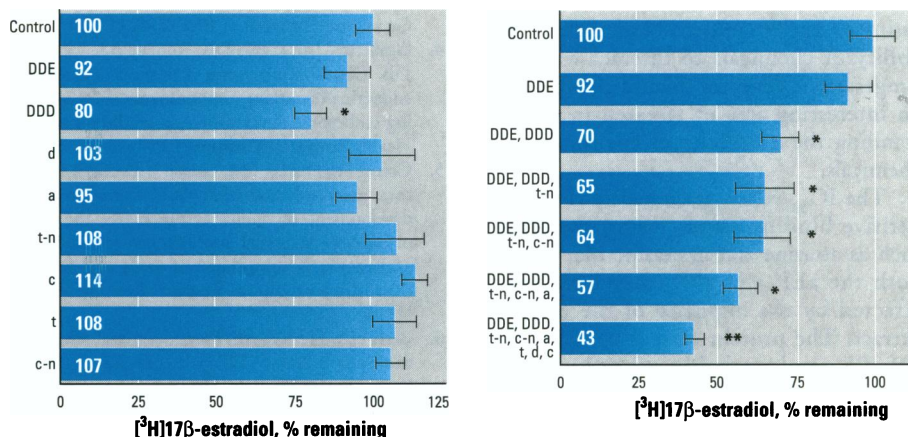DDT and its metabolites, with the exception of DDOH, did not compete

Figure 3. The effect of chemicals identified in Lake Apopka alligator eggs in the competition binding assay. An oviductal extract containing aER was incubated with [^{3}H]17β-estradiol with 18 μM *p,p'*-DDE (DDE), 2.6 μM *p,p'*-DDD (DDD), 0.63 μM dieldrin (d), 0.53 μM aroclor 1242 (a), 0.25 μM *trans*-nonachlor (t-n), 0.22 μM chlordane (c), 0.2 μM toxaphene (t), 0.16 μM *cis*-nonachlor (c-n) individually (A) or in combinations (B) at the same concentrations as used individually. The percent [^{3}H]17β-estradiol remaining is shown for each treatment. The data are the mean of three independent experiments with three replicates. Error bars indicate standard error. *p<0.05 indicates significant inhibition of binding compared to control value. **p<0.05 indicates significant inhibition of binding compared to control and treatments 1–4. No significant difference was observed between treatments 5 and 6.
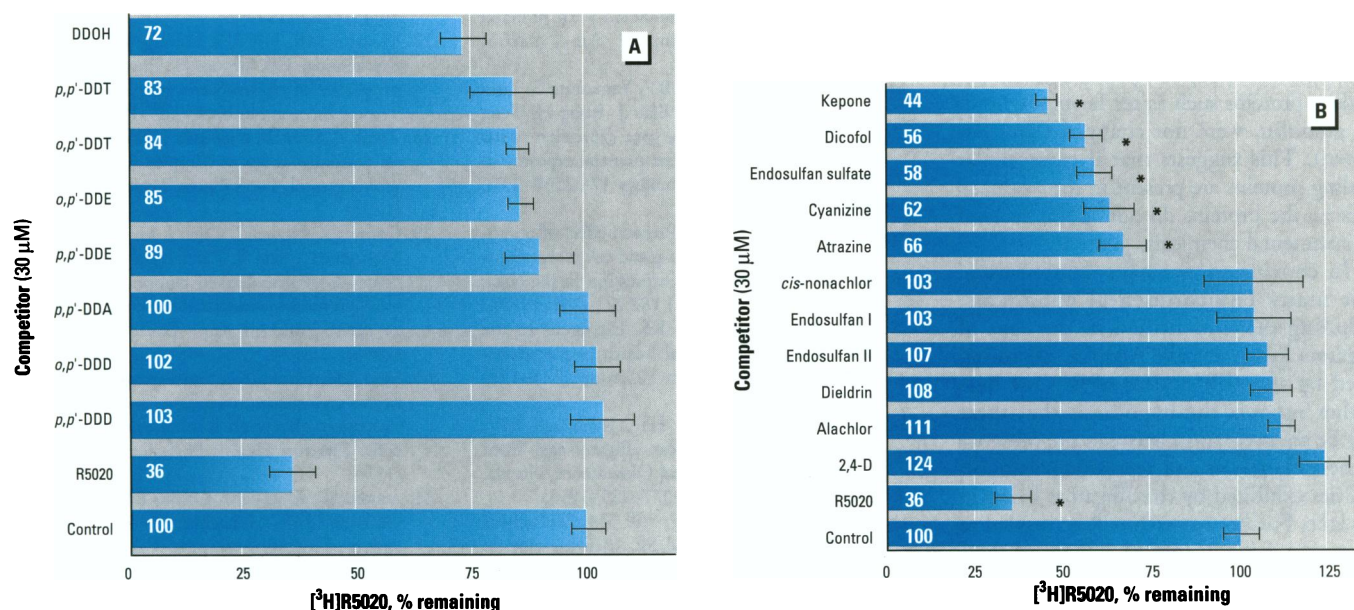

Figure 4. Competition binding assay with environmental chemicals with aPR. Abbreviations: DDOH, 2-2-*bis*(*p*-chlorophenyl)-ethanol; 2,4-D, 2,4-(dichlorophenoxy)acetic acid. An oviductal extract containing aPR was incubated with [^{3}H]R5020 with (A) 30 μM of DDT metabolites or (B) 30 μM of the indicated environmental chemicals. The percent [^{3}H]R5020 remaining is shown for each treatment. The data are the mean of three independent experiments with three replicates. Error bars indicate standard error. *p<0.05 indicates significant inhibition of binding compared to control value.

strongly for binding to the aPR. These data suggest the DDT metabolites may have a lower affinity for the aPR than for the aER. However, some of the modern-use chemicals, such as dicofol and cyanazine, competed with both [³H]17β-estradiol for binding to the aER and with [³H]R5020 for binding to the aPR. The ability of these chemicals to bind to the aER and aPR suggests that some chemicals may be promiscuous in interacting with steroid hormone receptors. The results with the aPR should be interpreted cautiously because it is not known if these chemicals function as progestins or antiprogestins. Nonetheless, the ability of chemicals to interact with the progesterone receptor should prove to be an interesting area of research in understanding the specificity of environmental chemicals.

The IC_{50} values generated in the competitive binding assays, using chemicals such as atrazine and cyanazine that bound both the aER and aPR, may have been affected by the impurity of the protein extract. The protein extract contains the ER, PR, and probably other hormone receptors such as the androgen receptor. Therefore, the measurement of the IC_{50} value of a chemical for the ER will be higher if the chemical also binds to other hormone receptors, effectively lowering the concentration of the chemical available to interact with the ER.

We have previously demonstrated that some environmental chemicals such as o,p'-DDT do not appreciably bind to extracellular binding proteins in serum (32). The IC_{50} values for some environmental chemicals, after purification of the oviductal extract with DNA-cellulose to remove extracellular binding proteins such as sex hormone-binding globulin, were not changed (data not shown). This suggests that if extracellular binding proteins are present in the oviductal extracts, the proteins do not appear to bind environmental chemicals.

In conclusion, we have demonstrated that many environmental chemicals, including those identified in the eggs of alligators living in Lake Apopka, are recognized by the aER and/or aPR. Our data further support the hypothesis that there may be an association between the presence of these chemicals and reproductive abnormalities exhibited by the alligators living in this lake. Further research will be needed to determine if the hormonal activity of these chemicals, as demonstrated in other species, is directly responsible for the reproductive abnormalities of the American alligator in Lake Apopka.

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